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(54) Title: <b>CARBOHYDRATE-MODIFIED POLYMER CONJUGATES WITH ERYTHROPOIETIC ACTIVITY</b>			
(57) Abstract  Biologically active conjugates of glycoproteins having erythropoietic activity and having at least one oxidized carbohydrate moiety covalently linked to a non-antigenic polymer are disclosed. Methods of preparing the conjugates and treatment methods employing the conjugates are also disclosed.			

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CARBOHYDRATE-MODIFIED POLYMER  
CONJUGATES WITH ERYTHROPOIETIC ACTIVITY  
CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of U.S. Patent Application Serial No. 672,696 filed on March 18, 1991, the disclosure of which is hereby incorporated by reference.

BACKGROUND OF THE INVENTION

The present invention relates to conjugates of glycoproteins having erythropoietic activity with non-antigenic polymers. The conjugates exhibit erythropoietic activities comparable to that of the corresponding non-conjugated glycoproteins.

EPO is a glycoprotein and a colony-stimulating factor regulating the formation of erythrocytes. Exogenous EPO is indicated in the treatment of anemic disorders, hematopoietic dysfunction disorders and related diseases.

The coupling of peptides and polypeptides to PEG and similar water-soluble polyalkylene oxides (PAO's) is disclosed by U.S. Patent No. 4,179,337. Physiologically active polypeptides modified with PEG exhibit dramatically reduced immunogenicity and antigenicity yet circulate in the bloodstream considerably longer than unconjugated proteins. The disclosure of this patent is incorporated by reference herein. Examples of such therapeutic protein conjugates include tissue plasminogen activator, insulin, interleukin II and hemoglobin.

To conjugate polyalkylene oxides, the hydroxyl end-groups of the polymer must first be converted into reactive functional groups. This process is frequently referred to as "activation" and the product is called an "activated polyalkylene oxide." In most instances, covalent attachment of the polymer is effected by reacting the activated polyalkylene oxides with  $\epsilon$ -amino moieties of lysine residues.

In several situations, polypeptide activity is reduced or lost when the  $\epsilon$ -amino moieties of the lysine residues are modified. Other proteins may have only a small number of available amino groups, and consequently  
5 very few polymer anchoring sites. As a result, many proteins of interest cannot be conjugated in this manner.

Both of the foregoing problems are typified by EPO. For example, human EPO only has eight free lysine  
10 residues having  $\epsilon$ -amino moieties available for polyalkylene oxide conjugation. In addition, as the degree of conjugation of these free lysine residues increases, the specific activity of the EPO decreases.

U.S. Patent No. 4,904,584 discloses  
15 polyalkylene oxide conjugation of lysine-depleted polypeptide variants, including EPO. The variants have lysine attachment sites and allegedly provide a more homogeneous and less random attachment of polyalkylene oxide chains. The disclosure of this patent is limited  
20 to covalent attachment of amino-reactive polyalkylene oxides to lysines. Such variants are rare and impractical. The applicants herein, however, have sought an alternative because substitution of lysines is expensive, time-consuming, and could effect homology of  
25 the protein, as well as in vivo activity. For example, PAO conjugation with lysines can alter the charge or isoelectric point of the protein. Variations in binding, activity and/or solubility between modified and unmodified proteins have been observed.

30 A means by which readily available EPO and other erythropoietic glycoproteins can be polyalkylene oxide conjugated without a loss of specific activity would be highly desirable.

#### SUMMARY OF THE INVENTION

35 It has now been discovered that long-acting conjugates of non-antigenic water-soluble polymers with erythropoietic glycoproteins can be prepared having a bio-activity comparable to that of native glycoproteins.

A key to this discovery is that covalent attachment of non-antigenic water-soluble polymers to oxidized carbohydrate moieties has little effect upon the erythropoietic activity of the glycoproteins.

5 Therefore, in accordance with the present invention, a biologically active conjugate is provided of a glycoprotein having the ability to effect erythropoiesis and having at least one oxidized carbohydrate moiety covalently linked to a non-antigenic  
10 polymer. The conjugate is preferably EPO or a glycoprotein having erythropoietic activity covalently attached to an activated form of a polyalkylene oxide such as polyethylene glycol. Polyethylene glycols having a molecular weight of about 5,000 are preferred.

15 The present invention also provides methods of preparing the conjugates. The methods include the step of reacting a glycoprotein having erythropoietic activity and having at least one oxidized carbohydrate moiety with an activated substantially non-antigenic  
20 polymer having a linking group for attaching the polymer to an oxidized carbohydrate. Thus, the non-antigenic polymer covalently attaches to the oxidized carbohydrate moiety of the glycoprotein. Preferred methods include reacting EPO with an activated form of a polyalkylene  
25 oxide such as a hydrazide, hydrazine, semicarbazide, thiosemicarbazide, amine or hydroxylamine activated polyalkylene oxide.

The invention also provides methods of treating anemia or other conditions associated with  
30 reduced endogenous erythropoietin or erythropoiesis. In this aspect of the invention, treatment includes administering an effective amount of the conjugates described herein to mammals requiring such therapy.

As a result of the present invention,  
35 conjugates having substantially prolonged erythropoietic activity in vivo are provided. These high activity conjugates are substantially resistant to in vivo hydrolysis, and thus require less frequent

administration and often lower dosages when compared to unmodified EPO and lysine-conjugates thereof.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

5 The conjugates of the present invention include glycoproteins having the ability to effect erythropoiesis, the formation of erythrocytes. These glycoproteins can be prepared or obtained from a variety of sources, including recombinant or mammalian extracted EPO. In one aspect, the EPO is prepared using  
10 recombinant techniques. The recombinant forms may be human or animal based, natural or mutant strains. In this regard, the recombinantly prepared EPO such as that disclosed in U.S. Patent Nos. 4,703,008 or 4,835,260 may be used herein. The disclosure of these  
15 patents are hereby incorporated by reference. The lysine depleted EPO disclosed by the above-cited U.S. Patent No. 4,904,584 may also be used herein. The disclosure of this patent is likewise hereby incorporated by reference. Alternatively, EPO may be  
20 obtained from mammalian sources such as human, bovine, ovine or porcine materials. See, for example, U.S. Patent Nos. 4,465,624 or 4,397,840, the disclosures of which are also hereby incorporated by reference.

The glycoprotein EPO has been characterized as  
25 a monomer weighing about 30,000 daltons having three possible glycosylation sites. The protein: carbohydrate ratio has been estimated to be about 3:1 suggesting that there are a number of sites that can be made available for polymer attachment upon oxidation of the  
30 carbohydrate moieties of the glycoprotein.

As used herein, the expression "the ability to effect erythropoiesis" means any glycoprotein, glycopolypeptide or portion thereof which demonstrates  
35 in vivo erythropoietic activity. These glycoproteins are prepared by using techniques known to those of ordinary skill in the art such as tissue culture, extraction from animal sources or by recombinant DNA methodologies. Transgenic sources of glycoproteins are

also contemplated. Such materials are obtained from transgenic animals, i.e., mice, pigs, cows, etc., wherein the glycoprotein is expressed in milk, blood or tissues. Insect sources of recombinant EPO are also contemplated. See, for example, Krantz, Blood, 77(3), 419-34 (February 1, 1991).

The non-antigenic water-soluble polymers included in the conjugates are preferably polyalkylene oxides. Within this group of substances are alpha-substituted polyalkylene oxide derivatives such as methoxypolyethylene glycols or other suitable alkyl-substituted derivatives such as C<sub>1</sub>-C<sub>4</sub> groups. It is preferred, however, that the non-antigenic polymer be a monomethyl-substituted PEG homopolymer. Alternative polyalkylene oxides such as other polyethylene glycol homopolymers, polypropylene glycol homopolymers, other alkyl-polyethylene oxides, bis-polyethylene oxides and co-polymers or blocked co-polymers of polyalkylene oxides are also useful.

The bis-activated polymers can be homo or heterobifunctional. Thus, the artisan can prepare cross-linked EPO conjugates or three-part conjugates containing EPO, a polymer and an additional substance which enhances bioactivity. Such substances include interleukins such as IL-3 or IL-6, growth factors, stimulating factors such as CSF, GM-CSF, and the like, or peptides or other moieties known in the art to enhance the activity of glycopolypeptides in vivo.

In those aspects of the invention in which PEG-based polymers are used, it is preferred that they have number average molecular weights between about 200 and about 100,000 daltons, and preferably between about 2,000 and about 20,000 daltons. A molecular weight of 5,000 daltons is most preferred.

Alternative non-antigenic polymeric substances include materials such as dextrans, polyvinyl pyrrolidones, polysaccharides, starches, polyvinyl alcohols, polyacrylamides or other similar non-

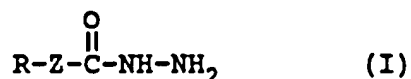
immunogenic polymers. Those of ordinary skill in the art realize that the foregoing is merely illustrative and unintended to restrict the type of non-antigenic polymers suitable for use herein.

5 One or more polymer chains are covalently attached to oxidized carbohydrate moieties of the glycoprotein by reacting suitably activated non-antigenic polymers with reactive carbonyl groups on oxidized carbohydrate moieties of the glycoprotein.  
10 Reactive carbonyl groups can be generated on saccharide units of carbohydrate moieties of glycoproteins, for example, by oxidizing vicinal diols of the carbohydrate moieties with excess periodate utilizing a reaction well understood by those of ordinary skill in the art, or  
15 enzymatically, e.g., by use of galactose oxidase. For purposes of the present invention, the reactive carbonyl group is defined as being either a ketone or aldehyde group, excluding other carboxyl-containing groups. Aldehyde groups are preferred, because they are more  
20 reactive than ketones.

Reactive carbonyl groups can be added to the glycoprotein, preferably to a targeted portion thereof, by reacting it with a polysaccharide functionalized to include a hydrazine, hydrazide, hydroxyamine,  
25 semicarbazide or thiosemicarbazide group. The glycoprotein is then oxidized with periodate to generate reactive carbonyl groups for conjugation with an activated polymer of the present invention.

"Suitably activated non-antigenic polymers" is  
30 understood by those of ordinary skill in the art to mean that the polymer is functionalized to include a group reactive with oxidized carbohydrate moieties, such as hydrazide.

Preferred hydrazide activated polymers of the  
35 present invention are acyl hydrazides having a structure corresponding to Formula I:



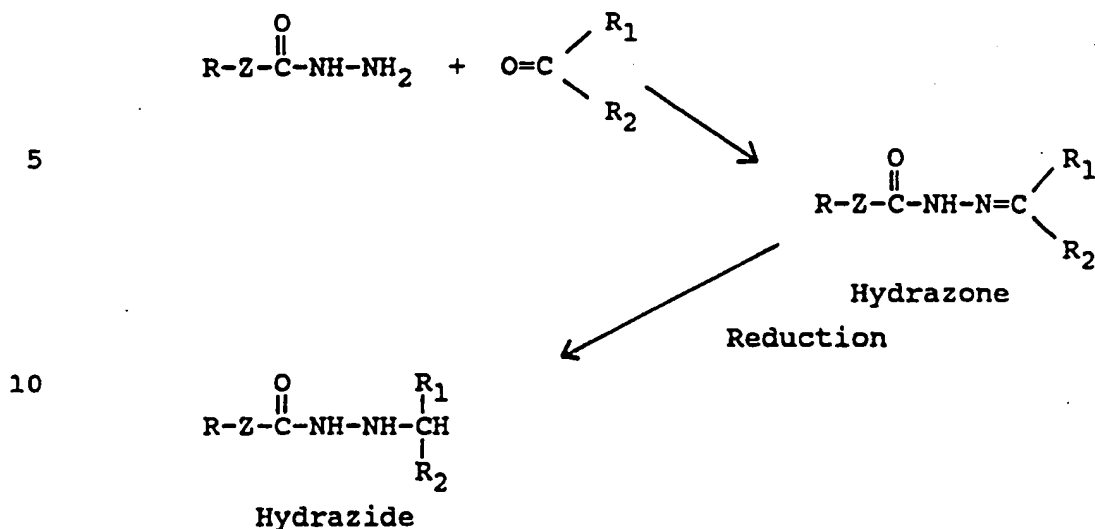


wherein R is one of the above-disclosed water-soluble non-antigenic polymers and Z is O, NH, S or a lower alkyl group containing up to ten carbon atoms. Z is preferably O, which forms a type of hydrazide linkage known as a semicarbazide linkage.

The semicarbazide derivative in which Z is O is prepared by reacting, for example, the methoxypolyethylene glycol-N-succinimide carbonate (SC-m-PEG) described in U.S. Patent No. 5,122,614 with an excess of hydrazine in an organic solvent in which the reactants are soluble, such as methanol, methylene chloride, toluene and the like. The disclosure of U.S. Patent No. 5,122,614 with respect to the preparation of methoxypolyethylene glycol-N-succinimide carbonate is hereby incorporated by reference. After the reaction is completed, the solvents and excess hydrazine are then removed utilizing conventional techniques.

The preparation of semicarbazide and other hydrazide polymer derivatives is described with reference to m-PEG for purposes of illustration, not limitation. Similar products would be obtained with any of the polymers suitable for use with the present invention, and it will be clear to those of ordinary skill in the art how this preparation can be adapted to the other suitable polymers.

The reaction of the acyl hydrazides of Formula I with a glycoprotein to form a hydrazone linkage is illustrated by the reaction sequence of Scheme 1 in which R and Z are the same as described above in Formula I and  $R_1$  and  $R_2$  form an oxidized carbohydrate moiety of a glycoprotein on which reactive carbonyl groups have been generated:

Scheme 1

15           The hydrazone can be reduced to the more stable alkyl hydrazide by reacting the hydrazone with, for example,  $\text{NaBH}_4$  or  $\text{NaCNBH}_3$ .

20           The process of the present invention includes preparing or providing the activated polymer and thereafter reacting it with a glycoprotein having erythropoietic activity and having at least one oxidized carbohydrate moiety. The carbohydrate moieties can be oxidized, for example, by reacting the glycoprotein in aqueous solution with sodium periodate or enzymatically

25           using galactose oxidase or combination of neuraminidase and galactose oxidase as disclosed by Solomon et al., J. Chromatography, 510, 321-9 (1990). The reaction runs rapidly to completion at room temperature. The reaction medium is preferably buffered, depending upon the

30           requirements of the glycoprotein. The oxidized glycoprotein is then recovered and separated from the excess periodate by column chromatography. Alternatively, the coupling reaction can be achieved by adding the activated polymer in situ before removing

35           excess periodate.

          The reaction is carried out in a buffer such as 0.1 M phosphate buffer at a pH of from about 5.0 to about 8.0 in the dark. The glycoprotein having at least

one oxidized carbohydrate moiety is reacted with an appropriate amount of the activated polymer, which is typically present in a several-fold molar excess over the glycoprotein. The polymeric excess will range from about 5 to about 500 molar ratio excess and preferably from about 50 to about 300 molar ratio excess of the polymer of the glycoprotein. The reaction is carried out at temperatures from about 0°C to about 28°C over time periods ranging from a few minutes to as long as 24 hours. Temperatures from about 2°C to about 22°C are preferred and time periods of around about one hour are sufficient to carry out the conjugation reaction.

Following the conjugation reaction, the desired product is recovered using known techniques and purified using column chromatography or similar apparatus, if necessary. Depending upon the reaction conditions, the conjugates have from about 1 to about 30 polymeric chains attached to the glycoprotein. By controlling the temperature, reaction time, pH and molar excess of the polymer reacted with the glycoprotein, for example, the artisan can tailor the number of polymeric chains attached. In addition, different activated polymers will also contribute to the degree of conjugation. Conjugates containing from about 2 to about 12 polymeric chains are preferred, while conjugates containing from about 4 to about 7 polymeric chains are most preferred.

In another aspect, the conjugates of the present invention may further include one or more non-antigenic polymer chains covalently attached to the  $\epsilon$ -amino moieties of the lysine residues of the glycoprotein and/or the carbonyl groups of the glycoprotein. Such conjugates thus include polymers attached at both oxidized carbohydrate moieties and lysines of the protein to further prolong circulating life.

The lysine conjugates may be formed by contacting the glycoprotein with an amine-reactive

activated non-antigenic polymer. A preferred amine-reactive polymer is the polyethylene glycol-N-succinimide carbonate (SC-PEG) disclosed by U.S. Patent No. 5,122,614. Alternatively, the activated  
5 polyalkylene oxides described in U.S. Patent No. 4,179,337 may be used.

Molar excesses of SC-PEG ranging from 5 to 200 fold are contacted with the glycoprotein under the conditions similar to those described above for  
10 conjugation with hydrazide activated polymers. Temperatures during the conjugation, however, are preferably less than room temperature, and about 0°C to 5°C. The appropriate molar excess to attain the desired degree of lysine conjugation can be readily determined  
15 by one of ordinary skill in the art without undue experimentation.

Preferably, the glycoprotein is first reacted with the SC-PEG after oxidation of the carbohydrate moieties, followed by the removal of the unreacted  
20 SC-PEG using conventional techniques. The oxidized carbohydrate moieties of the lysine-conjugated glycoprotein are then reacted with suitably activated polymers employing the process steps described above. However, a one-pot technique may also be employed in  
25 which the amine-reactive polymer and oxidized carbohydrate reactive polymer are simultaneously reacted with the oxidized glycoprotein. Alternative or further modifications such as cysteine residue modification or carboxyl modification are also contemplated. For  
30 example, PEG amine can also be used to react with oxidized carbohydrates to form a Schiff Base which also may be reduced in situ to a secondary amine.

Another aspect of the present invention provides methods of treatment for anemia or other  
35 hematopoietic disorders associated with low levels of or dysfunctional EPO. The method includes administering an effective amount of the compositions described herein to alleviate the condition. Those of ordinary skill in the

art will realize that the amount of a conjugate used in the method of the present invention will vary somewhat from patient to patient; however, conjugates capable of delivering from about 0.1 to about 500 micrograms of EPO per administration and preferably about 5 to about 100 micrograms of EPO are contemplated. The optimal dosing of the conjugate can be determined from clinical experience. Moreover, the dosage will also be dependant on the potency and pharmacokinetic profile of the EPO included in the conjugate. Factors such as patient weight, age, sex and physical condition will also affect the dosage. Nevertheless, one of ordinary skill in the art will be able to readily determine the appropriate dosage without undue experimentation.

Further in this regard, the amount of a conjugate administered in an amount that is sufficient to significantly increase hematopoiesis. The maximal dosage for humans is the highest dosage that does not cause clinically important side effects.

An important feature, however, is that by covalently linking the non-antigenic polymer and the glycoprotein as described herein by attaching the polymer chain to an oxidized carbohydrate moiety of the glycoprotein, not only are the conjugates substantially resistant to hydrolysis in vivo, the conjugates also possess erythropoietic activity comparable to that of the corresponding non-conjugated glycoprotein. The conjugates thus alleviate anemic conditions, for example, in vivo to a greater extent than prior art compositions.

The following non-limiting examples set forth below illustrate certain aspects of the invention. All parts and percentages are by weight unless otherwise noted, and all temperatures are in degrees Celsius.

#### EXAMPLES

##### MATERIALS

Methoxypolyethylene glycol (m-PEG) was obtained from Union Carbide. The solvents used, as well

as  $\text{NaCNBH}_3$  and  $\text{NaIO}_4$  were obtained from Aldrich Chemicals of Milwaukee, Wisconsin. The methoxypolyethylene glycol-N-succinimide carbonate (SC-m-PEG) was prepared as described in U.S. Patent No. 5,122,614 using m-PEG having a molecular weight of about 5,000.

#### EXAMPLE 1

##### Synthesis of mPEG-Beta Alanine-Hydrazide

m-PEG ( $\text{MW}_n$  5000, 100 g, 20 mmol) was dissolved in toluene (250 mL) and azeotropically dried for two hours under reflux. The solution was brought to 25°C, diluted with methylene chloride (50 mL) and then treated with phosgene (30 mL of 20% toluene solution, 56 mmol) overnight. The solvents and excess phosgene were removed by rotary evaporation under vacuum. The solid residue of polymeric chloroformate was dissolved in methylene chloride (90 mL) and treated with beta-alanine ethyl ester hydrochloride (6.1 g, 40 mmol) predissolved in methylene chloride (total volume 30 mL) followed by triethylamine (8.4 mL, 60 mmol). Approximately 30 minutes later, the solution was diluted with toluene (50 mL), filtered and evaporated to dryness. The crude product was dissolved in warm (50°C) ethyl acetate (500 mL) and filtered through celite. The filtrate was diluted with isopropanol to a total volume of 1000 mL and left overnight at 25°C to facilitate precipitation of the product. Another recrystallization of the product from isopropanol was performed. The yield of the dried m-PEG-beta-alanine ethyl ester was 98 g (95%).

The ester (62 g, 12 mmol) was then dissolved in pyridine (120 mL) and treated with hydrazine (12 mL, 0.375 mole) under reflux for six hours. The solution was rotary evaporated to dryness and the residue crystallized twice from isopropanol and dried in vacuo over  $\text{P}_2\text{O}_5$ . The yield was 60 g (97%).

The absence of free hydrazine in the product was ascertained by reverse-phase (C-18) thin-layer

chromatography in water/methanol (3:1) using TNBS spraying solution for detection.

#### EXAMPLE 2

##### Synthesis of m-PEG-Carbazate

5 SC-m-PEG (103 g, 20 mmol) was added to an anhydrous methanol solution containing hydrazine (16 g, 0.50 mmol). The reaction mixture was stirred for two hours and then dried in vacuo. The residue was then dissolved in methylene chloride and filtered. After  
10 drying, the filtrate was recrystallized and the final product was found to have an IR spectrum corresponding to PEG-carbazate. This structure was further verified with Carbon-13 NMR and proton NMR.

The product was found to be free of hydrazine  
15 by reverse-phase (C-18) thin-layer chromatography in water/methanol (3:1) using trinitrobenzene sulfonic acid (TNBS) spraying solution for detection.

#### EXAMPLE 3

##### Carbohydrate Modification Of EPO With 20 m-PEG- $\beta$ -Alanine Hydrazide

11.9 mg of EPO (human recombinant Chinese Hamster Ovary (CHO) cell culture), was dialyzed against 0.1 M phosphate-buffered saline buffer solution, pH 6.0 using a Centricon-10 (a product of the Amicon Corporation of Beverly, MA). Then, 200 mM sodium  
25 periodate was added to make 10 mM of final concentration, and the solution was stirred for one hour at 4°C in the dark. 480 mg of the PEG- $\beta$ -alanine hydrazide of Example 1 (250-fold molar excess) was added  
30 to the solution and the mixture was stirred at 4°C overnight. The unreacted m-PEG- $\beta$ -alanine hydrazide was removed by dialysis into a buffer solution having a pH of 7.0. The modification was verified by SDS-gel and size exclusion chromatography. The degree of  
35 conjugation was 6 to 7 PEG's per EPO molecule. The EPO activity was measured by colorimetric assay with DAI-K cells, a murine lymphoblastic cell line dependent on IL-3, GM-CSF and EPO for growth. The cells are grown in

IMDM containing 5% FCS and incubated at 37°C in 5% CO<sub>2</sub> in air. The assay time is 72 hours. The cell growth is monitored by MTT dye uptake.

#### EXAMPLE 4

5 In this example, the PEG-EPO conjugates were prepared in the same manner as in Example 3, except that the activated PEG carbazate of Example 2 was used in place of the beta-alanine derivative of Example 1. The conjugates showed a similar activity.

#### 10 EXAMPLE 5

In this example, the PEG-EPO conjugates of Examples 3 and 4 were further modified. In each case, the hydrazone bonds linking the polymer to the glycoprotein were reduced by using NaBH<sub>4</sub>. In particular, the solutions containing each of the  
15 respective conjugates were mixed with 0.1 molar NaBH<sub>4</sub> overnight at 4°C. Thereafter, each of the mixtures was dialyzed into 0.1 M phosphate buffer, pH 7, to remove the excess reagents.

20 Alternatively, the reduction step can be done immediately after conjugation. This way, NaIO<sub>4</sub>, the unreacted PEG and NaBH<sub>4</sub> can then be removed at the same time.

#### EXAMPLE 6

##### 25 Modification Of EPO With SC-m-PEG

In this example, the same recombinant EPO of Example 3 was conjugated with SC-m-PEG. 7.5 mg of the EPO in citrate buffer was dialyzed against 0.1 M sodium phosphate buffer solution, pH 7.0 using the  
30 Centricon-10. The final concentration of the EPO was about 3 mg/mL. A two-fold molar excess of the SC-m-PEG was added to the solution and the reaction mixture was stirred for one hour at room temperature. The reaction was quenched by adding 0.1 M glycine. The excess  
35 SC-m-PEG was removed by centrifugation. The m-PEG-EPO was purified on an anion exchange chromatography column. EPO activity was measured as in Example 3. The degree



of conjugation was two PEG's per EPO molecule and the activity was 11.6 pg/mL.

#### EXAMPLE 7

##### Modification Of EPO With SC-m-PEG

5 In this example, the recombinant EPO of Example 3 was conjugated with SC-m-PEG as in Example 6; however, 5.1 mg of the EPO was reacted with a four-fold molar excess of SC-m-PEG. The m-PEG-EPO was purified, the modification was checked and the EPO activity was  
10 measured as in Example 3. The degree of conjugation was four PEG's per EPO molecule and the activity was 26 pg/mL.

#### EXAMPLE 8

15 In this example, the circulating half-lives of three PEG-EPO conjugates prepared above (Ex. 3, 6 and 7) were compared to the recombinant EPO from which they were prepared in rats. Twelve rats about  $300 \pm 25$  g were used for this experiment. Three rats were injected i.p. with the native human recombinant EPO and three  
20 each were similarly injected with the m-PEG-EPO of Examples 3, 6 and 7 at a dose of 16 to 20 IU/kg. At various time points, the rats were bled and plasma prepared. The plasma was stored at 4°C until assayed. EPO activity was determined using the colorimetric assay  
25 described in Example 3. The half-lives ( $T_{1/2}$ ) and specific activities are reported in the following table, as well as the molecular weights, numbers of free lysines and numbers of m-PEG conjugates. As noted above, the molecular weight, and consequently the number  
30 of free lysines and number of m-PEG conjugates, were determined by SDS-gel and size exclusion chromatography.

Example	Molecular Weight	Specific Activity (Max <sub>1</sub> Cell Growth) (DA1-K Cells)	# Of Free Lysines	# PEG's	T <sub>1/2</sub>
Native EPO	30.4 KD	8.3 pg/ml	8	---	1.8 min.
3	60-65 KD	9.1 pg/ml	8	6-7	21.5 hrs.
6	40 KD	26.0 pg/ml	6	2	20 min.
7	50 KD	11.6 pg/ml	4	4	11.8 hrs.

As can be seen from the table, m-PEG-EPO conjugates prepared in accordance with the present invention have a substantial increase in circulating life when compared to native EPO. The conjugates of the  
5 present invention also have a substantially increased specific activity when compared to the lysine-conjugated EPO's, as expressed in terms of the concentration required to produce Max<sub>1</sub> cell growth.

As will be readily appreciated, numerous  
10 variations and combinations of the features set forth above can be utilized without departing from the present invention as set forth in the claims. Such variations are not regarded as a departure from the spirit and scope of the invention, and all such modifications are  
15 intended to be included within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A biologically active conjugate comprising a glycoprotein having erythropoietic activity and having at least one oxidized carbohydrate moiety covalently linked to a water-soluble non-antigenic polymer.  
5
2. The conjugate of claim 1, wherein said glycoprotein is selected from the group consisting of recombinant and non-recombinant mammalian erythropoietins.  
10
3. The conjugate of claim 1, wherein said polymer is a polyalkylene oxide.
4. The conjugate of claim 3, wherein said polyalkylene oxide is an  $\alpha$ -substituted polyalkylene oxide.  
15
5. The conjugate of claim 3, wherein said polyalkylene oxide is selected from the group consisting of polyethylene glycol homopolymers, polypropylene glycol homopolymers, alkyl-capped polyethylene oxides, bis-polyethylene oxides and copolymers or block copolymers of polyalkylene oxides.  
20
6. The conjugate of claim 5, wherein said polymer is a polyethylene glycol homopolymer having a molecular weight between about 200 and about 100,000.
7. The conjugate of claim 6, wherein said polyethylene glycol homopolymer has a molecular weight between about 2,000 and about 20,000.  
25
8. The conjugate of claim 7, wherein said polyethylene glycol homopolymer has a molecular weight of about 5,000.  
30
9. The conjugate of claim 1, wherein said carbohydrate moiety is covalently linked to said polymer by a hydrazide linkage.
10. The conjugate of claim 1, wherein said non-antigenic polymer is selected from the group consisting of dextran and polyvinyl pyrrolidones.  
35
11. The conjugate of claim 2, wherein said erythropoietins are of recombinant origin.

12. The conjugate of claim 2, wherein said erythropoietins are of non-recombinant origin.

13. The conjugate of claim 1, wherein said glycoprotein is of transgenic origin.

5 14. The conjugate of claim 1, wherein said conjugate comprises from about 1 to about 30 polymer chains attached to oxidized carbohydrate moieties of said glycoprotein.

10 15. The conjugate of claim 14, wherein said conjugate comprises from about 2 to about 12 polymer chains attached to oxidized carbohydrate moieties of said glycoprotein.

15 16. The conjugate of claim 15, wherein said conjugate comprises from about 4 to about 7 polymer chains attached to oxidized carbohydrate moieties of said glycoprotein.

20 17. The conjugate of claim 18, wherein said conjugate further comprises one or more polymer chains attached to  $\epsilon$ -amino moieties of lysine residues of said glycoprotein.

25 18. A method of preparing a conjugate having erythropoietic activity comprising reacting a glycoprotein having erythropoietic activity and having an oxidized carbohydrate moiety, with an activated substantially non-antigenic polymer capable of linking to said carbohydrate moiety, so that a carbohydrate linked glycoprotein-polymer conjugate is formed.

30 19. The method of claim 18, wherein said polymer is a polyalkylene oxide.

20. The method of claim 19, wherein said polyalkylene oxide is an  $\alpha$ -substituted polyalkylene oxide.

21. The method of claim 19, wherein said polyalkylene oxide is a polyethylene glycol.

35 22. The method of claim 21, wherein said polyethylene glycol is a methoxypolyethylene glycol.

23. The method of claim 21, wherein said polyethylene glycol is a hydrazine-activated polyethylene glycol.

5 24. The method of claim 18, wherein said reacting step comprises reacting said glycoprotein and said activated polymer in a molar ratio of from about 1:5 to about 1:500, respectively.

25. The method of claim 23, wherein said molar ratio is between about 1:50 and about 1:300.

10 26. The method of claim 18, further comprising reacting said carbohydrate-linked polymer conjugate with a second activated polymer capable of linking with  $\epsilon$ -amino moieties of lysine residues of said glycoprotein.

15 27. The method of claim 26, wherein said second activated polymer comprises a polyalkylene oxide.

28. A method of treating anemia comprising administering a therapeutically effective amount of the conjugate of claim 1.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/06098

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) : C07K 15/14, 17/08, 17/10; C12N 15/16; A61K 37/24, 39/385, 47/48

US CL : 530/395, 397, 399, 402, 411; 435/69.4; 514/8, 21; 424/280.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/395, 397, 399, 402, 411; 435/69.4; 514/8, 21; 424/280.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, 4,847,325 (SHADLE ET AL) 11 JULY 1989, see abstract, columns 2, 3, 9, 12-15.	1-28
Y	US, A, 5,103,039 (REARDAN ET AL) 07 APRIL 1992, see columns 3-4.	1-28
Y	US, A, 4,179,337 (DAVIS ET AL) 18 December 1979, see abstract, columns 1-3 and claim 2.	1-28
Y	US, A, 5,122,164 (ZALIPSKY) 16 June 1992, see columns 1-2.	1-28
Y	US, A, 5,089,261 (NITECKI ET AL) 18 February 1992, see columns 1, 2-5.	1-28
X Y	WO, A, 92/16555 (ZALIPSKY ET AL) 01 October 1992, see the entire article.	1-11, 13-16, 18-25

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

31 AUGUST 1994

Date of mailing of the international search report

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/06098

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CA, A, 2,003,743 (CAPON ET AL) 23 May 1990, see page 6.	1-28
Y	J. MILTON HARRIS, editor, "POLY(ETHYLENE GLYCOL) CHEMISTRY: BIOTECHNICAL AND BIOMEDICAL APPLICATIONS", published 1992 by Plenum Press (N.Y.), pages 347-370, see entire document.	1-28
Y	Proc. Natl. Acad. Sci., USA, Volume 83, issued April 1986, J. D. Rodwell et al, "Site-specific Covalent Modification of Monoclonal Antibodies: In vitro and In vivo Evaluations", pages 2632-2636, see entire article.	1-28
Y	Advanced Drug Delivery Reviews, Volume 6, issued 1991, M. L. Nucci et al, "The Therapeutic Value of Poly(ethylene glycol)-modified Proteins", pages 133-151, see entire article.	1-28
Y	Biocatalysis, Volume 2, issued 1989, Urrutigoity et al, "Biocatalysis in Organic Solvents with a Polymer-bound Horseradish Peroxidase", pages 145-149, especially pages 145 and 147.	1-28
Y	The Journal of Biological Chemistry, Volume 267, No. 11, issued 15 April 1992, M. Higuchi et al, "Role of Sugar Chains in the Expression of the Biological Activity of Human Erythropoietin", pages 7703-7709, especially pages 7703-7704.	1-28

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